Effects of Four Independent Low-Phytate Mutations on Barley Agronomic Performance

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ABSTRACT

The seed phosphorus storage compound phytic acid (*myo*-inositol-1,2,3,4,5,6-hea*kis*phosphate) is poorly utilized by nonruminant animals. Low Phytate (LP) crops, in which reductions of phytate are accompanied by increases in nutritionally available P, are in development and their utility will be enhanced by competitive agronomic performance. To assess the performance of LP barley (*Hordeum vulgare* L.), sets of sib lines that are homozygous wild type (WT), or homozygous for one of four independent *low phytic acid* mutations (*lpa1-1*, *lpa2-1*, *lpa3-1*, and M955), were developed via backcrosses to Harrington. The WT sibsets performed similarly to the Harrington parent, suggesting that the major variable in these experiments was the presence or absence of the LP alleles. Under irrigation, M955, which has an extreme reduction in phytate, was associated with reduced yield and percentage plump kernels; all mutations except *lpa2-1* were associated with reduced test weight. In rain-fed locations, all mutations were associated with reduced test weight and percentage plump kernels, and all except *lpa1-1* were associated with reduced yield. These results suggest that one component of yield loss in LP types is reduced stress tolerance. The performance of the *lpa*1-1 mutation, which appears to be aleurone specific, suggests a potential strategy to avoid this source of yield loss: the use of genotypes where the desirable effect is limited to a target tissue, in this case the aleurone layer.

THE MAJORITY of seed phosphorus (P) is stored as phytic acid (phytate; *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) (Lott et al., 2000; Raboy, 1997). Monogastric animals lack the phytase enzyme necessary to digest phytate, thus causing phytate-P to be unavailable for nutritional needs and, ultimately, to be excreted in feces primarily as phosphate (Leytem et al., 2004), where it can contribute significantly to surface and ground water pollution (Sharpley et al., 2003). Phytate also is an effective chelator of nutritionally important mineral cations and thus has the potential to contribute to mineral deficiencies (Ravindran et al., 1995). Development of feed grain with significantly less phytate coupled with a greater amount of digestible P would be desirable.

Low phytate mutants have been isolated in several cereal crop species (barley; rice, *Oryza sativa* L.; maize, *Zea mays* L.; and wheat, *Triticum aestivum* L.) and soybean, *Glycine max* (L.) Merr. (Hitz et al., 2002; Guttieri et al., 2004; Raboy et al., 2000, 2001; Rasmussen and Hatzack, 1998; Wilcox et al., 2000). These mutants produce seed that contain normal levels of total P but a significant fraction of the phytic acid has been replaced by inorganic P or by *myo*-inositol phosphates containing

five or fewer P esters. Feeding trials that include LP cereal grains have been conducted on swine, fish, and poultry. These studies have associated the LP trait with improved P and mineral availability, associated improvements in animal growth, and/or reductions in fecal P (Ertl et al., 1998; Spencer et al., 2000; Li et al., 2001a,2001b; Veum et al., 2002; Jang et al., 2003; Overturf et al., 2003; Thacker et al., 2003; Leytem et al., 2004).

Field evaluations of LP and wild-type (WT) maize containing moderate (65%) reductions of phytate showed that germination and stand establishment were not affected, but eight of 14 LP-WT hybrid pairs showed grain yield reductions associated with the LP trait; overall, LP lines averaged 94% of the WT grain yield (Ertl et al., 1998). Evaluations of LP soybean with similar reductions in phytate performed similarly, but the LP trait was associated with significant reductions in seedling emergence (Meis et al., 2003; Oltmans et al., 2005). Preliminary agronomic evaluations of several LP mutations in barley have suggested that agronomic performance is negatively correlated with the degree of phytate reduction (Raboy, unpublished data). This study was conducted to examine the agronomic performance of barleys homozygous for four independent LP mutations that result in phytate reductions ranging from 35 to 95% of wild type, when grown in irrigated or rain-fed production environments.

MATERIALS AND METHODS
Derivation of LP and WT Barley Lines

The four mutations studied for this report were generated by sodium azide treatment of 'Harrington', and their origins have been described in detail (Dorsch et al., 2003). M955, *lpa3-1* (formerly M635), and *lpa1-1* (formerly M422) have phytate reductions, respectively, of approximately 95, 65, and 50%, with a proportional increase in the amount of inorganic P. The mutant *lpa2-1* (formerly M1070) has a phytate reduction of approximately 40%, with proportional increases in a pool of nonphytate P that includes inositol phosphates with five or fewer phosphate esters (phytic acid has six phosphate esters per molecule) as well as inorganic P. Total P is unaltered for *lpa2-1*, *lpa3-1*, and M955; *lpa1-1* conditions a slight reduction in total P. Mapping studies have placed *lpa1-1* in chromosome 2H and *lpa2-1* in chromosome 7H (Larson et al., 1998). M955 and *lpa3-1* have been mapped to chromosome 1H; it is not known whether they represent mutations of separate loci or allelic variants of the same locus (Rolinsky, 2002).

The lines examined in this study were developed by backcrossing each mutant line to the wild-type (WT) parent, Harrington. The *lpa1-1* lines were derived from BC$_4$ populations, *lpa2-1* and *lpa3-1* from BC$_3$ populations, and M955 from BC$_2$ populations. Selections were based solely on the presence of the LP mutant allele in all backcross cycles except for the final one. For the final backcross cycle, F$_{2.3}$ lines were grown as 1.3-m headrows under irrigation at Aberdeen, ID, in 2000. Heads were selected at random from rows that were visually

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similar to Harrington. Heads homozygous for either the LP or the WT allele for each of the four mutations were identified on the basis of inorganic P assays of five individual kernels as described (Dorsch et al., 2003), and the remainder of the seed from each head was grown in 2001 as $F_{3.4}$ 1.3-m headrows at Aberdeen, ID. Individual headrows were selected on the basis of visual similarity to Harrington, harvested in bulk, and their LP or WT status verified as described above. For this study, six WT and six LP sib lines were selected from each of the populations containing either *lpa1-1, lpa2-1, lpa3-1,* or M955. Lines evaluated in 2002 and 2003 were $BC_xF_{3.5}$ and $BC_xF_{3.6}$ populations, respectively.

Test Sites

The 24 WT and 24 LP lines, plus the Harrington WT parent, were grown at Aberdeen and Tetonia, ID (2002 and 2003) and Soda Springs and Filer, ID (2003 only). These locations offer diverse environments and differ significantly with respect to elevation above sea level (Filer, 1064 m; Aberdeen, 1338 m; Soda Springs, 1763 m; Tetonia, 1794 m), growing season, temperatures, and water availability. The growing season for Filer was late March to mid-July, and at Aberdeen early April to early August. For both locations, daily low to high temperatures during anthesis and grain filling generally ranged from 10 to 15°C to 27 to 35°C. The growing seasons for the Soda Spring and Tetonia locations were mid-May to late August, and temperatures during anthesis and grain filling were approximately 3 to 5°C cooler than for Aberdeen and Filer.

Prevailing agronomic practices for the growth of spring malting barley were used at all locations, including the use of sprinkler irrigation at Aberdeen and furrow irrigation at Filer. No irrigation was provided at Soda Springs or Tetonia. At the University of Idaho Research and Extension Center in Aberdeen, barley was planted on as part of a 3-yr rotation of potatoes-green manure (oat)-barley. Soil tests at Aberdeen showed similar levels of N, P, and K in both years (approximately $40 \mu g \ g^{-1}$ or $180 \ kg \ ha^{-1}$ N in the top 60 cm and $20 \mu g \ g^{-1}$ P and approximately $255 \mu g \ g^{-1}$ K in the top 30 cm); no additional fertilizer was added. At the University of Idaho Research and Extension Center in Tetonia, barley was planted as part of a 2-yr small grains-fallow rotation. Soil tests at Tetonia showed similar levels of N, P, and K in both years (approximately $2 \mu g \ g^{-1}$ or $9 \ kg \ ha^{-1}$ N in the top 60 cm and $40 \mu g \ g^{-1}$ P and approximately $255 \mu g \ g^{-1}$ K in the top 30 cm). No additional P or K was added. Nitrogen was added as a combination of ammonium nitrate and ammonium sulfate to bring available N to $100 \ kg \ ha^{-1}$. At Filer, barley was planted on a commercial farm following sweet corn. Soil tests at Filer showed $38 \mu g \ g^{-1}$ N (or $170 \ kg \ ha^{-1}$) N, $20 \ kg \ ha^{-1}$ P, and $230 \mu g \ g^{-1}$ K; $12.3 \ kg \ ha^{-1}$ N and $58.2 \ kg \ ha^{-1}$ P_2O_5 were added as monoammonium phosphate. At Soda Springs, barley was planted on a commercial farm as part of a continuous malt barley operation with no rotation; no soil tests were conducted. Fertility management was based on long-term averages of previous soil tests, and the precipitation, fertility management practices, and grain yield and quality at that site in the previous year. Fifty-six kilograms per hectare N and $22.4 \ kg \ ha^{-1}$ P_2O_5 were added as a combination of ammonium nitrate, ammonium sulfate, and monoammonium phosphate.

Experimental Design and Statistical Analysis

The experimental design at all locations was a randomized complete block with two replicates. Plots were planted with a small-plot drill (Hege) equipped with double disc openers in ranges of 10 side-by-side plots, 2.4 m in length, consisting of

7 rows on 17.8-cm centers, with approximately 36 cm between plots. Planting rates at Aberdeen and Filer were approximately $112 \ kg \ ha^{-1}$ and at Tetonia and Soda Springs approximately $90 \ kg \ ha^{-1}$. The outside plots of each range were bordered by other plots that were not part of the test. Each range was separated by a 1.2 m alley. Harvesting was done with a small plot combine (Wintersteiger). Agronomic data were collected for days to heading, plant height, percent lodging, grain yield, grain test weight, and the percentage plump kernels (defined as kernels retained on a 2.38- by 19.1-mm slotted screen).

All data were analyzed by SAS v. 8.0 Proc GLM (1999. SAS Institute Inc., Cary, NC, USA). Subset (*lpa1-1, lpa2-1, lpa3-1,* or M955), genotype (WT or LP), and environment (irrigated or rain-fed), and their interactions were considered to be fixed effects. Subenvironments (locations and years) within environments and all other sources of variance were considered random. Comparisons of WT sibsets to Harrington were based Dunnett's comparisons of sibset × environment means, with sibset × subenvironment(environment) as the error term. Comparisons of LPA vs. WT were made using single-degree-of-freedom contrasts. All declarations of significance were based on $p < 0.05$.

RESULTS

The irrigated locations, Aberdeen and Filer, provided good, low-stress growing conditions as determined on the basis of visual observations and as evidenced by high mean grain yield, test weight, and percentage plump kernels (Table 1). The growing seasons of 2002 and 2003 were characterized by extreme drought, both in terms of short-term (current season) precipitation as well as long-term drought (depletion of soil moisture reserves from a lack of precipitation in prior years). Detailed precipitation records are not available for the Soda Springs location; however, the general precipitation patterns were widespread and data recorded at the Tetonia location is representative of the southeastern Idaho region which includes Soda Springs. For Tetonia, precipitation averages recorded on-station for the period 1971 through 2000 were 510 mm annually, with an average of 185 mm recorded for the growing season (May–August). For 2001, the annual precipitation was only 302 mm. For

Table 1. Agronomic performance for selected characteristics of wild type (WT) and low phytate (LP) sibsets, and Harrington, grown under irrigation at Aberdeen (2002–2003) and Filer (2003), ID.

Sib Set	Phenotype	Lodging	Yield	Test weight	Plump kernels†
		%	kg ha⁻¹	kg m⁻³	%
lpa 1-1	LP	49	8487	658***	85
	WT	47	8320	675	88
lpa 2-1	LP	34***	8271	671	82
	WT	55	8429	676	83
lpa 3-1	LP	49*	7991	652***	82
	WT	59	7994	673	85
955	LP	26***	7147***	639***	77***
	WT	46	8253	672	89
Harrington	WT	55	8582	680	87
Location mean			8164	666	84

* Indicates significant difference in LP vs. WT values at $p = 0.05$ significantly different from Harrington check performance ($p = 0.05$).
*** Indicates significant difference in LP vs. WT values at $p = 0.01$ significantly different from Harrington check performance ($p = 0.05$).
† Percentage of kernels remaining on a 2.38- by 19.1-mm screen.

2002 and 2003, annual/growing season precipitation was, respectively, 430/116 mm and 385/59 mm. Thus, the Tetonia and Soda Springs locations imposed significant water stress which was more extreme at the Soda Springs site because it had not been fallowed the previous year. Visual symptoms of stress became obvious from the booting stage onward and included such symptoms as afternoon wilting, lack of seed set in late tillers, and a generally poor appearance. Consistent with these observations, the plants grown under rain-fed environments showed low yields, test weights, and percentages plump kernels (Table 2).

Analyses of variance showed substantial environmental interactions that could be traced primarily to differential responses of the LP sibsets to the irrigated vs. the rain-fed environments. Within environments, subenvironment (locations × years) interactions with sibset or genotype (WT or LP) were negligible. Variation among lines within a sibset was not a significant source of variability (e.g., all LP M955 lines or all *lpa1-1* WT lines). The interaction between LP sibsets and genotype was significant for most analyzed traits. Accordingly, presentation and discussion of the data are based on comparisons of sibset × genotype interaction means separately for the irrigated and rain-fed environments (Tables 1 and 2).

The WT sibs for each mutation were not significantly different than Harrington for any of the measured traits, in both the irrigated and rain-fed environments. This suggest that the source of any observed differences between the backcross-derived WT and LP sibs was the presence or absence of the mutant alleles. Therefore, the effects of each LP mutation could be assessed on the basis of WT vs. LP sib comparisons.

Performance under Irrigated Conditions

No differences for seedling emergence, stand establishment, and general appearance throughout the growing season could be detected on the basis of visual observations of the Harrington recurrent parent, WT lines, and LP lines. Analyses of heading dates and plant heights did not detect significant differences among LP sibsets nor between LP or WT genotypes. Mean heading dates for Aberdeen and Filer were 169 and 158 d after 1

January, respectively. Mean plant heights for Aberdeen and Filer were 94 and 107 cm, respectively. The *lpa2-1* LP sibset was not different from its WT sibset for yield, test weight, or percentage plump kernels (Table 1). There was a significant reduction in percentage lodging, which was surprising given the equal heights and yields of the WT and LP sibsets. Visual observations did not detect morphological differences that might explain this reduction. LP sibsets containing the *lpa1-1* and *lpa3-1* mutations showed relatively small changes when compared with their respective WT sibsets. No differences were detected for yield or percentage plump kernels between WT and LP sib lines, and for *lpa1-1,* no differences were detected for lodging. Both sibsets showed significant and similar reductions of test weight (Table 1).

Mutant M955, which conditions a 90% reduction in phytate, as compared with the 35 to 70% reductions conditioned by the other mutants, clearly had a negative impact on agronomic performance (Table 1). Substantial reductions in grain yield and percentage plump kernels were recorded, and the reduction in test weight was more severe than for *lpa1-1* or *lpa3-1*. Lodging percentage was reduced also, most likely as a consequence of the significantly reduced yield.

Performance under Rain-Fed Conditions

No differences for seedling emergence or stand establishment were detected on the basis of visual observations of the Harrington recurrent parent, WT lines, and LP lines for any of the mutant classes. However, the LP sibsets, particularly for *lpa2-1, lpa3-1,* and M955, tended to appear less vigorous and exhibit more signs of stress than their respective WT sibsets or Harrington. These differences were slight and could not consistently be noted. No differences could be detected for heading dates and plant heights among LP sibsets nor were any detected between the LP or WT genotypes. The mean heading date for Tetonia was 194 d after 1 January; heading dates were not recorded at Soda Springs because the frequency of observations was insufficient; however, qualitative assessments conducted at several dates surrounding head emergence also suggested that all lines had similar heading dates. Mean plant heights for Tetonia and Soda Springs were 48 and 45 cm, respectively.

Additional data for agronomic performance under rain-fed conditions are shown in Table 2. The mutant *lpa1-1* yielded well, but the LP sibset had lower test weight and percentage plump kernels relative to the WT sibset. In contrast, mutants *lpa2-1, lpa3-1,* and M955 were associated with significant, and substantial, reductions in grain yield. The LP sibsets for all three mutations showed significant reductions in test weights and percentages plump kernels.

DISCUSSION

The materials developed for this study, and the diverse conditions under which they were tested, provided a comprehensive evaluation, in the background of Harrington, of the agronomic effects of four independent mutations

Table 2. Agronomic performance for selected characteristics of wild type (WT) and low phytate (LP) sibsets, and Harrington, grown under rain-fed conditions at Tetonia (2002–2003) and Soda Spring (2003), ID.

Sib Set	Phenotype	Lodging	Yield	Test weight	Plump kernels†
		%	kg ha^{-1}	kg m^{-3}	%
lpa 1-1	LP	0	1718	600***	65***
	WT	0	1935	625	72
lpa 2-1	LP	0	1321***	604***	47***
	WT	0	1728	614	56
lpa 3-1	LP	0	1354***	599***	52***
	WT	0	1836	619	64
955	LP	0	1162***	592***	49***
	WT	0	1769	620	70
Harrington	WT	0	1802	620	66
Location mean			1625	610	60

*** Indicates significant difference in LP vs. WT values at $p = 0.01$.
† Percentage of kernels remaining on a 2.38- by 19.1-mm screen.

conditioning low phytate in barley grain. The limited number of backcross cycles used to develop the LP and WT sib lines did not produce truly near-isogenic lines, but the representation of each phenotypic class by six sib lines should have produced good estimates of the characteristics of each population. Empirical support for this statement is provided by the recovery—for traits that showed changes—of the recurrent parent performance in the WT sib populations for each mutant class.

One limitation of these data is that the effects of the mutant alleles were studied in a single genetic background. However, all of these mutations have been evaluated in several other genetic backgrounds as part of an extensive effort to produce commercially competitive LP cultivars (Bregitzer, unpublished data). Selections for good agronomic appearance within early generations of recombinant LP populations—including two- and six-rowed lines, hulled, and hulless lines—grown at Aberdeen have consistently favored lines derived from crosses to *lpa1-1*, *lpa2-1*, and *lpa3-1*. For instance, of 140 single plant selections from the cross M955/86Ab2626//HB317 on the basis of vigorous plant appearance and plump kernels, only six (4%) were homozygous for the LP trait. Lines developed from all of these selections had very poor yields and small kernels. In contrast, selection among populations segregating for the other three mutations, the ratio of LP/LP:LP/WT:WT/WT plants closely approximates the expected segregation ration of 1:2:1. Yield trials of lines derived from *lpa1-1* have shown their yields to be generally higher than those derived from *lpa2-1* or *lpa3-1*. For instance, preliminary yield trials at Aberdeen in 2002 lines derived from backcrosses of *lpa1-1* to 'Colter' showed their mean yield to be 92% of Colter, compared with 66% for lines derived from *lpa2-1*. Lines developed from backcrosses of *lpa2-1* and *lpa3-1* to 'Stander' yielded, respectively, 100% and 82% of Stander. More extensive testing of one line, 00ID1550 (Colter*3/*lpa1-1*), showed that relative to Colter the mean yields under irrigated conditions (6 location-years) and rain-fed locations (6 location-years) was, respectively, 101 and 97%. Mean test weight for both irrigated and rain-fed conditions was 98% of Colter, consistent with the test weight reductions noted for *lpa1-1* in this study. Thus, it is reasonable to conclude that, at least on a qualitative basis, the results of this study may be predictive of those that would be obtained for these mutations when tested in other genetic backgrounds. Further studies will be necessary to verify this conclusion.

It was expected that the alterations of phosphorus profiles of the LP sibsets examined for this study would be similar to their mutant parent, under both the irrigated and rain-fed production environments. Previous characterizations of the original mutant lines, using seed produced in two different environments, Aberdeen, ID, and Saskatoon, SK, Canada, (Dorsch et al., 2003; Leytem et al., 2004; Overturf et al., 2003; Thacker et al., 2003), showed similar results with respect to the alterations in phosphorus profiles conditioned by these four mutations. Furthermore, semiquantitative analyses of seed from recombinant breeding populations for Pi con-

tent generally resulted in the ranking *lpa2-1* equal to or slightly less than *lpa1-1*; populations containing *lpa3-1* have slightly higher Pi content, and M955 populations have the highest Pi content. Qualitative evaluations of total P and Pi were conducted on grain produced in this study that confirmed this expectation and will be published elsewhere. In brief, the these studies showed Pi/total P concentrations (in mg g^{-1}) for the LP sibsets for Harrington, *lpa1-1, lpa2-1, lpa3-1*, and M955 to be 0.20/3.31, 1.06/3.00, 0.89/3.61, 1.86/3.49, and 2.63/3.54, respectively, for irrigated environments and 0.28/3.94, 1.05/3.39, 1.27/4.43, 1.89/4.04, and 3.04/4.24, respectively, for rain-fed environments. Statistical analyses revealed the following relationships: for Pi, Harrington $<$ *lpa1-1* = *lpa2-1* $<$ *lpa3-1* $<$ M955; for total P, *lpa1-1* $<$ Harrington = *lpa2-1* = *lpa3-1* = M955.

The data produced in this study indicate that the development of agronomically competitive cultivars carrying the *lpa1-1*, *lpa2-1,* or the *lpa3-1* mutations will be possible, but only the *lpa1-1* mutation may be compatible with the production of LP barley grain under conditions of moisture stress that are typical of rain-fed production environments, even those located in relatively cool, high altitude locations that are otherwise suitable for barley production. Because of the reductions in test weight associated with either *lpa1-1* or *lpa3-1*, the development of cultivars with acceptable test weight may be facilitated by introgressing these alleles into genotypes with relatively higher test weights, e.g., two-rowed types typically have significantly higher test weights than six-rowed types.

The reaction of lines to moisture stress suggest that the LP mutations are associated with significant perturbations of essential physiological functions in the whole plant, in addition to the desirable changes in seed P chemistry they condition. Phytate is the most abundant representative of a class of compound referred to as inositol phosphates (Shears, 2004). Both *myo*-inositol (the backbone of phytic acid) and inositol phosphates including phytate play important roles in hormonal responses in plants, and in signal transduction and stress response in many eukaryotes (Flores and Smart, 2000; Ishitani et al., 1996; Lemtiri-Chlieh et al., 2003; Perera et al., 2004). Not surprisingly, lines containing the mutant with the most extreme phenotype (M955; 90% reduction of phytate) fared relatively worse than lines carrying less extreme mutations.

Interestingly, *lpa1-1*, which had the best performance under moisture stress, has been shown to have a tissue-specific phenotype (Ockenden et al., 2004). Phytic acid has been shown to accumulate primarily in aleurone tissues, with lesser amounts accumulating in the germ (O'Dell et al., 1972). The *lpa1-1* mutation only affects P and phytate P in the aleurone layer. Phytic acid P levels in *lpa*1-1 germ are equal to or greater than wild type, whereas each of the other three mutations affect both the germ and aleurone layer. Although a causal relationship has yet to be established, and the impact of *lpa1-1* on whole plant processes has not yet been studied, it is not surprising that a mutation limited to a specific seed tissue might have more impact on grain quality than on grain

yield. This restricted tissue specificity of barley *lpa1-1* represents a potential strategy to engineer low phytate crops without significant yield reductions; i.e., to development of germplasm where the block in phytic acid synthesis is restricted to the aleurone.

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